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Diphtheria Toxoid-Antitoxin Floccules

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The precipitate that forms when diphtheria toxin combines with antitoxin has been shown to be antigenic by Sordelli and Serpa (1925), Hartley (1925), Schmidt and Scholz (1926), Eberhard (1926) and Glenny, Pope, Waddington and Wallace (1926). Toxin-antitoxin floccules (T.A.F.) have been used successfully for active immunisation in the human subject without giving rise to the nonspecific reactions that frequently result from the injection of toxin-antitoxin mixtures (T.A.M.) or of toxoid. Hartley (1926) showed that the floccules produced from mixtures containing a minimum quantity of antitoxin were the best antigens while the amount of nitrogenous material present was only one fiftieth of that in the original mixture.

There are objections to the use of floccules produced by the combination of antitoxin with unmodified toxin. It is well known that under certain unusual conditions toxin-antitoxin mixtures may become toxic, and it has been our practice for some years past to prepare all mixtures intended for human use from toxin modified to toxoid. There may conceivably be also conditions in which toxin-antitoxin floccules may become toxic owing to a relatively greater destruction of antitoxin than of toxin. There can be no such danger in the use of floccules prepared from modified toxin. Hartley has shown that the most toxic floccules are the most efficient; by the use of toxoid, floccules can be prepared with a minimum quantity of antitoxin without any limitation to dosage as would be necessary if the floccules were toxic.

In a preliminary experiment, suspensions of floccules were prepared from toxin and from toxoid using the same serum and the same relative degree of neutralisation. Animal experiments showed that toxoid-antitoxin floccules were as efficient antigens as those prepared from unmodified toxin. In the next experiment Hartley's work on toxin-antitoxin floccules was confirmed with toxoid floccules. Three different batches of formalin toxoid were used and from each batch three preparations of floccules were made using (*a*) the minimum amount of antitoxin producing flocculation within three days, (*b*) the amount causing the most rapid flocculation (Lf mixture) and (*c*) excess of antitoxin. The floccules were washed three times and suspended in phenol saline to one tenth of the original volume. Guinea-pigs were injected subcutaneously with 1·0 c.c. and 5·0 c.c. quantities and

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injected intracutaneously with Schick toxin each week commencing three weeks after the initial injection. Table I. recording the results of the experiment gives the serial numbers of the Schick injections first yielding a negative reaction, *i.e.* the immunity index.

TABLE I.

Showing the immunity index for a series of toxoid-antitoxin floccules prepared from different batches of toxoid with different amounts of antitoxin.

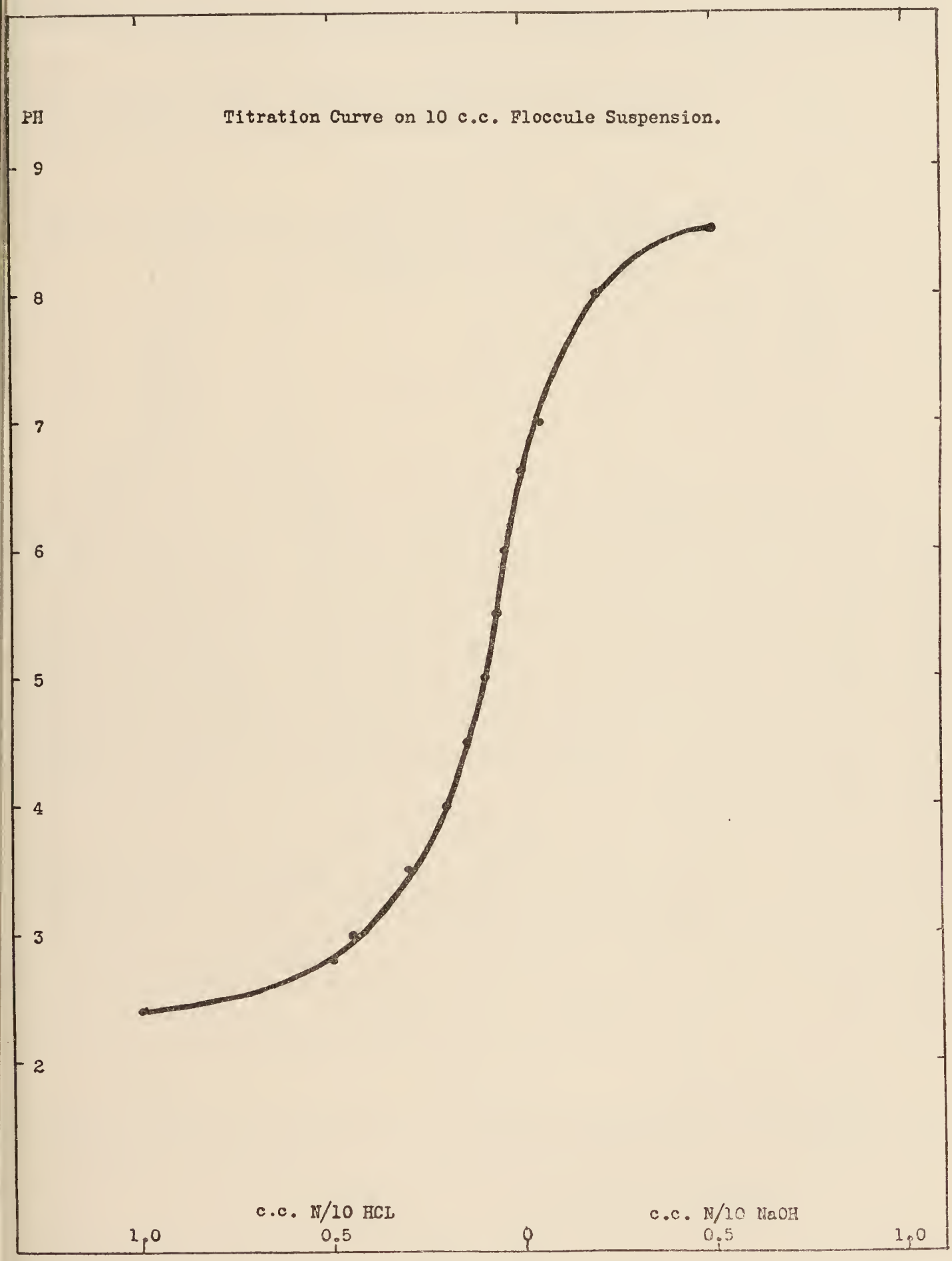
Toxoid.	Lf value.	Units of antitoxin per c.c. of toxoid.	Immunity index.					
			1·0 c.c.			5·0 c.c.		
A	20	14	2	3	3	3	4	4
		20	5	7	...	3	3	6
		32	4	7	...	9	..	...
B	9	6	2	3	5	2	3	5
		9	4	...	...	2	3	3
		12	over 10	...	...	...	...	...
C	14	10	2	...	...	2	3	4
		14	5	over 6	10	2	3	over 4
		20	6	7	over 10	4	7	over 10

It will be seen from table I. that toxoid-antitoxin floccules prepared with the minimum amount of antitoxin are better antigens than those prepared with more antitoxin. It will be further seen that with these floccules doses of 1·0 c.c. and of 5·0 c.c. appear equally efficient, thus confirming the earlier work of Glenny, Pope, Waddington and Wallace (1926). In this connection it must be pointed out that the "immunity index" method measures the rapidity of production of immunity rather than degree of immunity produced. The amount of antitoxin produced and the degree of tolerance to toxin ultimately reached by the guinea-pig injected with 5·0 c.c. doses may be higher. It appears reasonable to suggest that rapidity of immunisation in the human subject as indicated by a negative Schick reaction is of more importance than the attainment of a high level of immunity.

Further experiments were devised to explore methods of improving the antigenic values of the floccules. Ramon (1923) recovered antitoxin by freeing toxin floccules from salt, treating them with dilute acid and heating for one hour at 58° to 60°. For our work it was necessary first to determine the optimum *p*H for the removal of antitoxin. Acid or alkali was added to a series of quantities of a suspension of toxoid-antitoxin floccules to adjust them to different hydrogen ion concentrations. Owing to the unbuffered nature of the material it was necessary first to plot the accompanying titration curve electrometrically; the quinhydrone electrode with the direct reading *p*H meter described by Pope and Gowlett (1927) was used. This curve determined the amount of acid or alkali to be added to the larger samples which were then checked electrometrically; by this method

nly small adjustments were necessary to bring the material to the equired *p*H. After 24 hours each batch was filtered and the clear uid adjusted to *p*H 7·0 and its antitoxic content determined (table II.).

It will be seen from table II. that toxoid-antitoxin floccules dissolve



completely when the *p*H is adjusted to 2·5 and almost completely dissolve at *p*H 3·0; upon adjusting the filtrate to *p*H 7·0, material is again precipitated but a considerable amount of antitoxin is left in solution. The maximum recovery of antitoxin is obtained from floccules at *p*H 3·5 but a considerable amount is recovered from floccules at *p*H 4·0 although they appeared insoluble and no precipitate appeared

on adjusting the *p*H of the filtrate to 7·0. The floccules again become soluble at a *p*H of about 10. This region was not however fully investigated in the present work.

TABLE II.

Showing the amount of antitoxin recovered from toxoid-antitoxin floccules submitted to different hydrogen ion concentrations.

*p*H.	Appearance after 24 hours.	Appearance of filtrate adjusted to *p*H 7·0.	Antitoxic content in units per c.c.
2·5	Clear	Cloudy	2·0
3·0	Slightly opalescent	,,	6·0
3·5	Opalescent	Almost clear	25·0
4·0	Undissolved	Clear	4·0
4·5	,,	,,	0·8
5·0	,,	,,	0·1
5·5	,,	,,	0·01
6·0	,,	,,	0·002
7·0	,,	,,	0·002
8·0	,,	,,	0·01
9·0	,,	,,	0·02

In the next experiment a suspension of floccules was adjusted to *p*H 4·0, the highest hydrogen ion concentration at which they appeared insoluble. After 24 hours the floccules were centrifuged and the supernatant liquid was found to contain 10 units of antitoxin per c.c. The residue was washed at *p*H 4·0 and then suspended in saline at *p*H 8·0. After 24 hours they were again centrifuged and the supernatant liquid and a suspension of the residue in saline tested upon guinea-pigs for antigenic value in parallel with the original suspension of floccules.

TABLE III.

Showing the antigenic index of toxoid-antitoxin floccules after removal of some antitoxin by the action of acid.

	Immunity index.					
	1·0 c.c.			5·0 c.c.		
Original suspension	1	1	3	2	3	5
Supernatant of suspension at *p*H 8·0 of acid floccules	2	3	3	2	3	over 4
Final suspension of acid floccules .	1	2	...	1	...	...

Table III. shows that floccules submitted to the action of acid are to some extent soluble in saline when suspended at *p*H 8·0. In spite of the removal into solution of some antigenic material the washed suspension of acidified floccules was at least as efficient as the original material, but too few animals were available to determine whether acidification and consequent removal of antitoxin improved the antigenic value.

In view of the remarkable heat stability of toxoid, an endeavour as made to destroy antitoxin in the floccules by heat. A suspension f floccules was heated for one hour to temperatures of 50° 60° 70° 80° nd 100° and then injected into guinea-pigs. The results recorded in able IV. show that toxoid-antitoxin floccules are improved in antigenic fficiency by heating for one hour at 80°. Subsequent work still in rogress has shown that a considerable part of the antigen is in olution after the floccules are heated.

TABLE IV.

Showing the antigenic index of toxoid-antitoxin floccules after heating to different temperatures.

	Antigenic index.					
	1·0 c.c.			5·0 c.c.		
Original—unheated . . .	2	3		1	2	6
Heated 1 hour at 50° . . .	2	2	3	2	3	3
,, ,, 60° . . .	3	3	3	2	3	3
,, ,, 70° . .	2	2	2	2	2	2
	2	2	3	2	2	4
,, ,, 80° . . .	1	1	2	1	1	
,, ,, 100° . . .	2	3	3	1	1	3

The results here recorded are sufficient to warrant the recommendation that T.A.F. for human use should be prepared from toxoid and not from toxin, and to suggest that the preparation can be improved by removing some of the antitoxin by chemical or physical means.

There is scope for considerable experimental work to determine the optimum condition for modifying the floccules not only along the lines already suggested but also by making use of the most suitable type of antitoxin. Owing to the different ratios between the *in vitro* and *in vivo* values of different sera, flocculation occurs only when toxin is mixed with a large excess of one type of antitoxin but if another type is used the toxin must be in excess. Table V. shows the amount of

TABLE V.

Showing the amount of toxin or of antitoxin in excess in 1 *c.c. of flocculating mixtures of a given toxin with three different types of antitoxin.*

	Antitoxin used in mixtures.		
	A.	B.	C.
Mixtures containing minimum antitoxin	7 units of antitoxin	1250 M.R.D.s of toxin	50,000 M.R.D.s of toxin
Lf mixtures	18 units of antitoxin	0·1 unit of antitoxin	5000 M.R.D.s of toxin
Mixtures containing maximum antitoxin	55 units of antitoxin	1·0 unit of antitoxin	1·0 unit of antitoxin

toxin or antitoxin in excess in mixtures of the same toxin with three types of antitoxin. The mixtures tested were made with the minimum amount of antitoxin causing flocculation within 3 days, the amount causing the most rapid flocculation and the maximum amount.

It may be mentioned that not only can toxoid replace toxin in floccules but also that toxoid can be treated with sodium ricinoleate without losing its antigenic properties. Larson and Eder (1926) claim that toxin so treated can be used for human immunisation without giving rise to non-specific reactions. There is no reason to suppose that toxoid so treated would not be equally free from non-specific effect and would certainly be free from the distinct danger of the toxin becoming unmasked.

Conclusion.

1. Toxoid-antitoxin floccules are as good antigens as toxin-antitoxin floccules.

2. The use of toxoid frees the preparation from all possible danger of increase in toxicity.

3. The antigenic efficiency of the floccules is increased by heating to 80° C. for one hour.

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